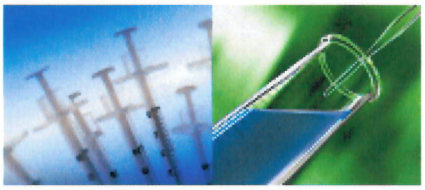

nPOD
Network for Pancreatic Organ Donors with Diabetes

ISOLATION OF PBMC

1 PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to outline procedures for isolation and cryopreservation of peripheral blood mononuclear cells (PBMC).

2 SCOPE

This SOP will be applied to whole blood that is separated to cells suitable for cell culture.

3 RESPONSIBILITIES

3.1 Managers and supervisors are responsible for making sure that technicians are properly trained and equipment and facility are maintained in good working order.

3.2 Laboratory personnel are responsible for reading and understanding this SOP and related documents and to perform these tasks in accordance with the SOPs. They are responsible for following clinical laboratory and tissue banking best practices.

4 EQUIPMENT and MATERIALS

The materials, equipment and forms listed in the following list are recommendations only and alternative products as suitable may be substituted for the site specific task or procedure.

Refrigerated table top centrifuge (Eppendorf Centrifuge 5810 R)
Cryovials with O-rings (FisherSci, Cat. No. 12-565-163N)
CryoStor CS10 cell freezing media (BioLife Solutions, Cat. No. 07930), store at 4°C
Dulbecco's Phosphate Buffered Saline (D-PBS), Mg^{2+} Ca^{2+} free (Invitrogen, Cat. No. 10010-023), with antibiotic/antimycotic added prior to use at a final concentration of 1% anti/anti in D-PBS, store at 4°C
Antibiotic-Antimycotic Solution (Anti/Anti) , 10,000 I.U./ml Penicillin 10,000 ug/ml Streptomycin 25 ug/ml Amphotericin B (Corning, Cat. No. 30-004-CI), aliqouted in 5 mi and store at -20^{0}C
Transfer pipettes, 5 ml, 10ml, 25 ml serological pipettes, serological pipetter (Pipet-Aid or equivalent), pipettes (1000 ul, 200 ul, 20 ul) and sterile filter tips
70% ethanol (EtOH)
Ficoll-Paque Plus (GE Healthcare)
10-50% Clorox Bleach
0.17M Ammonium chloride solution (Stemcell, Cat. No. 07800), store at 4°C
Cell counting supplies: pipettes and tips (1000 ul, 200 ul, 20 ul Cellometer (Nexcelom), disposable cell counting chamber (Nexcelom), Cellometer AOPI Staining Solution in PBS (Nexcelom, Cat. No. CS2-0106-5ML), store at 4°C
CoolCell freezing container (VWR, Cat. No. 95059-860)
CoolCell Filler Vials, 2ml (Biocision, Cat.No. BCS-3105)

5 SAFETY

5.1 Use universal safety precautions when handling human samples and personal protective equipment (e.g., face mask with shield, gloves, lab coat or apron). Dispose of all solutions and supplies in contact with human blood in biohazardous waste.

6 PROCEDURE

6.1 Record number of un-coagulated blood tubes received, tube color (for anticoagulant), and blood volume in the case worksheet (see SOP Case Processing).

6.2 Mix tubes by gently inverting 5 to 8 times. Sterilize tubes and caps with 70% EtOH and move to biosafety hood.

6.3 Pour up to 20 ml whole blood in a 50 ml sterile conical tube. Discard empty blood tubes in biohazardous sharp waste container.
Add room temperature D-PBS with 1% anti/anti to the conical tube to a final volume of 35 ml and resuspend.

6.4 Add 15 ml of Ficoll to a second 50 ml sterile conical tube. While holding the tube containing Ficoll at a 30° angle, slowly pipette whole blood/PBS solution so that it overlays the Ficoll at a rate of about 10 ml/minute.

6.5 Centrifuge at 450 x g for 30 minutes at room temperature, **WITHOUT BRAKE,** and maximum acceleration.

6.6 Aspirate upper layer to within 1 cm of the interphase layer and discard.

6.7 Collect interphase layer (buffy coat) using a transfer pipette. Slowly sweep the top of the desired layer with constant suction and transfer to a sterile 50 ml conical tube.

6.8 Discard the remaining material in the appropriate liquid biohazardous waste container inside the hood.

6.9 Add D-PBS with anti/anti to the collected buffy coat to a final volume of 35 ml for the first wash.

6.10 Centrifuge at 430 x g for 10 minutes at room temperature with maximum brake and acceleration.

6.11 Aspirate and discard the supernatant.

6.11.1 If red blood cells are present, the pellet will be red.

6.11.2 Re-suspend the pellet in 1 ml D-PBS with anti/anti by gently pipetting and add 9 ml 0.17M ammonium chloride per 1 ml cell pellet. Incubate the cells at room temperature for 5-7 minutes.

6.12 Add D-PBS with anti/anti to a total volume of 30 ml and centrifuge at 430 x g for 7 minutes at room temperature with maximum brake and acceleration.

6.13 Discard the supernatant and re-suspend the pellet in 1 ml D-PBS with anti/anti, then repeat step 6.12.

6.14 Aspirate supernatant completely and re-suspend in 1 ml D-PBS with anti/anti. Add 9 ml D-PBS with anti/anti for a final volume of 10 ml.

6.14.1 Immediately after re-suspending the cell pellet, transfer 20 ul aliquot of the cell suspension to microfuge tube and perform a cell count, as described below in 6.16.

6.15 Meanwhile, centrifuge the remaining cell suspension at 430 x g for 7 minutes at room temperature with maximum break and acceleration. After centrifuging, use a sterile pipette and remove the supernatant without disturbing the pellet. Discard the supernatant into biohazardous liquid waste container.

6.15.1 A suitable re-suspension volume is detailed below in 6.18 after obtaining a cell count.

6.16 Cell Counting

6.16.1 Set up a clean Cellometer disposable cell counting chamber by removing protective coating on both sides of the chamber. Add 20ul of Cellometer AOPI Staining Solution in PBS to the 20 ul cell suspension aliquot and mix gently.

6.16.2 Load 20 ul of cells into the sample introduction port and place chamber in Cellometer.

6.16.3 Follow the assay protocol for "Immune cells, low RBC" and adjust bright image field focus if necessary. Save final cell count and viability data for entry in sample database.

6.17 Data Calculations

6.17.1 Cell viability(%) = (No. of vialble cells counted)/(total cells counted)x100%.

6.17.1.1 Expected cell viability is >75% and is usually > 90%.

6.17.2 Cell concentration: cells/ml = (total live cells/4) x dilution factor (DF) x 10^4

6.17.3 Total cells: (cells/ml) x suspension volume (ml)

6.18 Calculate suitable re-suspension volume to provide 1 x 10^7 cells/ml final cell concentration (optimal range is 1x 10^7cells/ml but can be increased to up to 3x10^7 cells/ml).

6.19 Cell cryopreservation. Work quickly and keep tube on ice. Gently flick the tube with finger several times and resuspend the pellet by adding the first 1 ml of freezing media drop by drop, very slowly, while rapidly rotating the 50 mL conical tube in a circular motion. Gently resuspend cell suspension and slowly add rest of cell freezing media according to cell count.

6.20 Aliquot the cell suspension into labeled cryotubes (see SOP Case Processing) in 200 ul-1 ml volumes. Avoid exposure of cells to freezing media longer than 15 minutes before starting the cryopreservation to improve post-thaw cell viability.

6.21 Immediately place cryovials in CoolCell freezing containers and place in a -80°C freezer overnight. Inset CoolCell Filter Vials into empty wells of CoolCell freezing containers when freezing less than a full batch of vials to ensure the desire rate of-1^{0}C/minute.

6.22 The following day, transfer the vials from CoolCell to storage boxes to the liquid nitrogen cryotank. Store at vapor phase. See SOP Cryotank Use and Maintenance.

6.23 Record cryovial storage location, aliquot volume, and cell concentration in sample inventory database.

6.24 Quality control measures include periodic review of cell viabilities and yields and data calculations.

7 REFERENCES

7.1 Mallone R., et al. *Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses*: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clinical and Experimental Immunology. 2010.

7.2 SOP 53 Cryotank Use and Maintenance

7.3 SOP 57 Case Processing

7.4 SOP 60 Isolation of Cells from Spleen, Thymus, and Lymph Nodes

8 REVISION HISTORY

Version	Date	Revision
1	8/28/12	IK updated cell freezing media catalog number
2	6/15/15	MP updated equipment and reagent list, and cell counting process

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